Comparison of immune responses induced by porcine parvovirus virus-like particles and inactivated vaccine

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Abstract: Laboratory-prepared inactivated porcine parvovirus (PPV) vaccines and VP2 virus-like particles (VLPs) were utilized to immunize gilts. PPV BQ strain and SP2/0 cells were used. Hemagglutination-inhibiting (HI) antibody titers were measured in the immunized gilts and the differences in cytokine production of interferon gamma (IFN-γ, IL-2 and IL-4) were compared. $CD4^+$ and $CD8^+$ T cells proliferation were compared by flow cytometry. The variation between the immune response level induced by inactivated PPV vaccine and VP2 VLPs were determined. The results showed that all vaccinated gilts had HI antibody titers reaching 1:256 for at least one month post immunization and the peak level of antibody could be sustained for one month; further, PPV antibodies could be detected in the second week post immunization with VP2 VLPs. We also found that the level of cytokines (IFN-γ, IL-2 and IL-4) were all increased post immunization and continued to rise after the booster immunization; the level of increase in IFN-γ and IL-2 were significantly higher than IL-4. The flow cytometry results showed that the numbers of the $CD4^+$ and $CD8^+$ T cells subsets were significantly higher in the groups immunized with inactivated PPV vaccine or VP2 VLPs than those of negative control group ($p<0.01$); additionally, the number of $CD4^+$ cells in the gilts that received VP2 VLP immunization was significantly higher than the inactivated vaccine group ($p<0.01$). In summary, the inactivated PPV vaccine and PPV VP2 VLPs were both able to induce humoral and cellular immunity, but the VP2 VLPs lead to better cellular immune responses in gilts compared to those of the inactivated vaccine.

Keywords: Virus-like particles, inactivated vaccines, cellular immunity, humoral immunity.

INTRODUCTION

Porcine parvovirus (PPV) was first isolated from sows in Germany by Mayr *et al* and belongs to the genus Parvovirus of family Parvoviridae (Hong *et al*., 2010). Porcine parvovirus (PPV) infection is a major disease leading to reproductive disorders in sows like stillbirths, malformed fetuses, mummified fetuses and sick piglets. Occasionally, PPV infection also causes dermatitis, diarrhea and arthritis (Eicio *et al.*, 2012). In 1987, Jie Wu *et al*. first reported mixed infections of PPV and Japanese encephalitis virus in China. Conditions such as mixed infections of PPV and porcine circovirus (PCV2), mixed infections of PPV and porcine reproductive and respiratory syndrome virus (PRRSV) have also been reported (Ellis *et al.*, 2000; Kennedy *et al.*, 2000; Allan *et al.*, 2000). Because PPV only occurs as one serotype and is highly immunogenic, vaccination against PPV is an effective way to prevent PPV infection and improve the reproductive rate of sows. There are more than 10 countries that have developed PPV vaccines. Most of these vaccines are inactivated vaccines, which have the advantages of having good safety features, complete antigenic components and high immunogenicity. Suzuki and Fujisaki (1976) were the first to develop inactivated vaccines for the prevention and control of PPV infection. Virus-like particles (VLPs) are empty capsid structures that self-assemble from viral capsid proteins or from viral capsid proteins and envelope proteins. The morphology of VLPs is similar to that of natural viral particles. VLPs can effectively stimulate humoral and cellular immune responses and have high immunogenicity. In addition, VLPs do not contain genetic material and have good safety features. To date, researchers have developed corresponding VLPs for more than 30 different types of viruses infecting humans and animals, of which some have become candidate vaccines for the prevention of viral infections (Noad & Roy, 2003; Zhang *et al.*, 2009; Zhou *et al.*, 2010). Vaccination has been an effective way to reduce the disease resulting from PPV infection so this study utilized an inactivated vaccine developed based on the PPV BQ-C strain that was previously prepared in our laboratory and purified PPV VP2 VLPs that were expressed *in vitro* using the baculovirus expression system.

MATERIALS AND METHODS

Virus, cells, serum and animal

The PPV BQ-C strain with a titer of $10^{6.0}$ $TCID_{50}$/mL was

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used for the virulent challenge assay, ST cells and recombinant baculovirus AcNPV-VP2, as well as PPV positive and negative sera, were all kept in the department of swine infectious diseases at the Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences. Inactivation was performed in 1% BEI at 32°C for 24 h; 3-5-month-old healthy gilts were purchased from a pig farm in Harbin City, Heilongjiang Province.

Vaccine

The PPV BQ-C strain was inoculated onto PK-15 cells monolayers and cultured in medium containing 10% fetal bovine serum (FBS) for 48 h at 5% CO_2 and 37°C (Zhang *et al.*,2010). Once 80% of the cells showed a cytopathic effect (CPE), the cells were collected, and the viral titer was determined to be 2^{11}. The viruses were then inactivated in formaldehyde at 37°C for 12 h to generate inactivated antigens, and the titer was measured to be 2^9. Inactivated antigens that were tested (by HI test) and met the standard were mixed with the adjuvant Montanide ISA 15A VG (SEPPIC, France) at a 1:1 ratio for emulsification to generate the inactivated PPV vaccine (García-Piñeres *et al*., 2007). Tests for physical traits and sterility were conducted for the prepared inactivated vaccines in accordance with the requirements for biological products.

AcNPV-VP2 was inoculated at 10% to a 70%-80% confluent monolayer of Sf9 cells in Grace medium with 2% FBS. The cells were collected after 96 h of culture at 27°C by centrifugation at 2,000 rpm and 4°C for 30 min. The cells were washed twice using PBS at a volume of 1/10 of the original culture medium volume and then resuspended. After cell lysis, the lysate was supplemented with protease inhibitors and kept on ice for 30 min. The lysate was mixed once every 10 min by inversion and then centrifuged at 12,000rpm for 20min. The supernatant was collected, and the recombinant protein was purified according to the His· Tag fusion protein purification operation manual of MERCK. The immunization reagents of the VP2-VLP group were emulsified with the adjuvant Montanide ISA 15A VG according to a protein: adjuvant ratio of 85:15.

Immunization of the glits

Each of the two prepared vaccines was used to immunize five healthy gilts that were three to five months old and negative for PPV antibodies. The vaccines were delivered by intramuscular injection in the neck, followed by one booster immunization three weeks later. The doses of the PPV inactivated vaccine and VLPs were both 2 mL/gilt (1 mg/mL), and five negative control gilts were also established. The animals were grouped according to the different immunization conditions and were reared under the same conditions. Blood samples were collected weekly after immunization to isolate serum.

Virulent challenge

When the immunized gilts completed the fifth week of gestation, we selected three gilts with satisfactory levels of antibodies and three control gilts, which were then raised in separate groups under the same conditions. The gilts were challenged with the PPV BQ-C strain with a titer of $10^{6.0}$ $TCID_{50}$/mL at a dose of 4mL/pig, of which 2 mL was injected intramuscularly and 2mL was applied intranasally. The clinical response and the conditions of the sows and fetuses of the challenged sows were observed.

Hemagglutination assay

Blood samples were collected weekly after immunization to isolate serum to detect the antibody production levels and the changes in antibody level duration. Serum PPV antibodies were detected using the HI method and were tested according to a previously reported method (Send *et al.,* 1986).

Detection of cytokines

The procedures were conducted according to the manuals of the cytokine detection kits (IFN-γ, IL-2 and IL-4) (BD Biosciences, USA).

Flow cytometry

A total of 100μL of diluted $CD4^+$ or $CD8^+$ antibody solution (1:80 dilution, BD Biosciences, USA) was taken to resuspend fresh blood, and the suspension was carefully mixed and kept in the dark for 20 min. One milliliter of hemolysin was added to the cell mixture, mixed well and kept at room temperature in the dark for 15-20 min, until it was observed that the liquid became clear and transparent. The mixture was centrifuged at 1,200 rpm for 5 min at room temperature. The centrifuge tube was inverted to discard the supernatant, and the remaining liquid around the edge of the tube was removed by absorbent paper. Each sample was supplemented with 2 mL of PBS, mixed well, centrifuged at 1,200 rpm for 5 min at room temperature, and then washed once again. Each sample was supplemented with 500 μL of PBS and mixed well. The results were measured in a Beckman flow cytometer within 1 h and then saved.

Ethical consideration

This study was approved by the Ethics Committee of the Harbin Veterinary Research Institute of CAAS, PR China.

RESULTS

Detection of HI antibody

Blood samples were collected weekly from immunized gilts, and the HI antibodies were measured using the kaolin method as shown in fig. 1. The test results showed that for the inactivated PPV vaccine, PPV antibodies could be detected in the first week post the first immunization; the antibody titer rapidly increased to 2^{15}

in the second week after immunization, and the high antibody level lasted until the thirteenth week (91 d) at the end of the test. For the VP2 VLPs, PPV antibodies could be detected in the second week post the first immunization; the antibody titer was rapidly increased to 2^{15} in the third week post immunization, and similarly, the high antibody level sustained until the thirteenth week. Upon virulent challenge in the seventh week, the antibody levels showed temporary reductions in both immunization groups and increased again in the second week after challenge, indicating that both vaccines can protect against viral infection. Post the challenge, the antibody titer of the control group was increased to some extent. The titer of the negative control group remained at a low, stable level during the whole process.

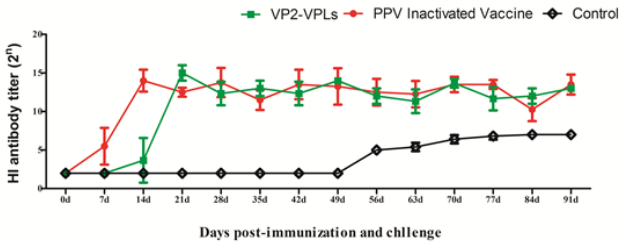

Fig. 1: Antibody titers of immunized gilts before and after virulent challenge

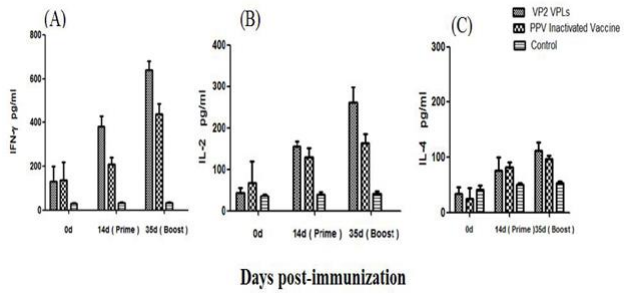

Fig. 2: Levels of cell factor from the serum of the immunized gilts

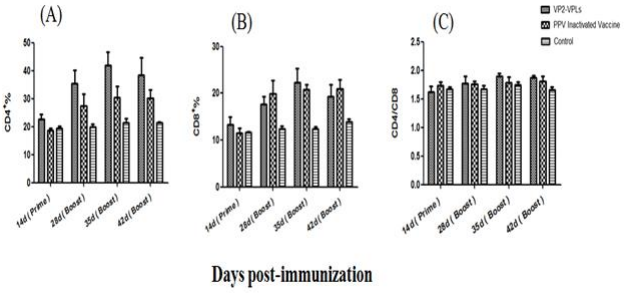

Fig. 3: Changes in the $CD4^+$ and $CD8^+$ T cell subpopulations in the peripheral blood

Detection of cytokines

Blood samples were collected from the gilts before the first immunization, 14 d after the first immunization and 14 d after the booster immunization (35 d) to detect the cytokines IFN-γ, IL-2 and IL-4 (fig. 2). The IFN-γ detection results (fig. 2A) showed that the IFN-γ secretion level was already significantly increased 14 d after the first immunization compared with the pre-immune level; the IFN-γ secretion level in each immunization group was increased again 14 d after the booster immunization (35 d). The IL-2 detection results (fig. 2B) showed that the IL-2 secretion level was increased somewhat after the first immunization; however, the IL-2 levels of the two immunized groups were both below the high secretion level of IFN-γ. The IL-4 test results (fig. 2C) showed that the IL-4 secretion level was increased somewhat after the first immunization but that the increase was slow; the IFN-γ and IL-2 secretion levels were significantly higher than that of IL-4. In both immunization groups and at each time period, the IFN-γ level of the VP2 VLP immunization group was significantly higher than that of the inactivated PPV vaccine group ($p<0.01$).

Flow cytometry results

Peripheral blood was collected from the immunized gilts 14 d after the first immunization and 28, 35 and 42 d after the booster immunization; T lymphocyte cell subtypes were detected using flow cytometry, followed by statistical analysis of the data. The flow cytometry analysis results (fig. 3) showed that the numbers of $CD4^+$ (fig. 3A) and $CD8^+$ (fig. 3B) T cell subtypes in gilts immunized with VP2 VLPs and inactivated vaccine were significantly higher than those in the negative control group ($p<0.01$); further, the number of $CD4^+$ cells in the gilts immunized with VP2 VLPs was significantly higher than that of the inactivated PPV vaccine group. In addition, the $CD4^+/CD8^+$ ratios of all of the test groups (fig. 3C) were in the normal range (1.5-2.0).

DISCUSSION

PPV infection is an important reproductive disorder in sows. Currently, there is no cure for this disease, and vaccine immunization is the primary prevention measure used to control this disease. Because PPV can cause significant damage in sows and breeding herds, sow stocks in large modern pig farms in China as well as breeding herds and gilts in breeding farms are required to receive PPV prophylactic immunization. Therefore, there is a large demand for a PPV vaccine. However, most current vaccines are limited to cultured pathogens or recombinant proteins, which mainly induce a humoral immune response, and it is difficult to induce the cellular immune responses required for the prevention and treatment of chronic diseases (Guillen *et al.*, 2010). VLPs can not only stimulate the body to produce humoral immunity but also generate cellular immune responses. This study utilized laboratory-prepared inactivated PPV vaccine and VP2 VLPs to immunize gilts and compared the cellular immunity level various between inactivated PPV vaccine and VP2 VLPs and further explored the immunological effects of the two vaccines.

In terms of humoral immunity, HI antibody levels can reflect the protection capacities of immunization reagents. The HI test results in this study showed that PPV antibodies could be detected in the first week after the first immunization with inactivated PPV vaccine; the antibody titer rapidly increased to 2^{15} in the second week after immunization, and the high antibody level lasted until the thirteenth week (91 d) at the end of the test. For the VP2 VLPs, PPV antibodies could be detected in the second week after the first immunization; the antibody titer rapidly increased to 2^{15} in the third week after immunization, and similarly, the high antibody level lasted until the thirteenth week. Upon virulent challenge in the seventh week, the antibody titer of the control group was increased to some extent. The virulent challenge test showed that both vaccines could prevent viral infection.

In terms of cellular immunity, this study detected and analyzed the cytokine levels of IFN-γ, IL-2 and IL-4. It was clearly observed that the three cytokines were significantly increased in both immunization groups compared with the pre-immune levels. However, the IFN-γ and IL-2 secretion levels were higher than the IL-4 levels. Production of IL-2 and IFN-γ is characteristic of the T helper 1 (Th1) - mediated immune response, which is necessary for the production of cytotoxic T lymphocytes (CTLs). The characteristic Th2 response includes the production of IL-4 and IL-10, which can induce B cell differentiation (Pinto *et al.,* 2006; Orsolic *et al.,* 2005). Therefore, the cytokine detection results suggest that the two vaccines used in this study are both biased toward the Th1-type immune response.

The levels of T lymphocyte-secreted cytokines are one of the indicators of cellular immunity, and the proportions of $CD4^+$ and $CD8^+$ T lymphocytes and the $CD4^+/CD8^+$ ratio are key parameters of the immune system (Dhur *et al.*, 1991). $CD4^+$ T cells differentiate into two phenotypes: Th1 cells, which stimulate immune-responsive cells to mediate immune signals, and Th2 cells, which stimulate humoral responses or allergic reactions (Constant & Bottomly, 1997). $CD8^+$ T cells can induce the death of infected somatic cells or tumor cells, and these T cells kill virus-infected cells (or other cells infected with other pathogens). $CD8^+$ T cells can also eliminate damaged or dysfunctional cells. The flow cytometry results in this study indicate that immunization using VP2 VLPs or inactivated vaccine could both lead to the proliferation of $CD4^+$ and $CD8^+$ T cell subsets in gilts; further, the cell numbers were significantly higher than those of the negative control group. In addition, the $CD4^+/CD8^+$ ratio was elevated in the two groups of immunized gilts. The $CD4^+$ T cell number in gilts immunized with PPV VLPs was significantly higher than that in the inactivated vaccine group, suggesting that antigen-induced immune responses mainly consisted of a $CD4^+$ cell-based immune response. This result indicates that in gilts, VP2 VLPs can induce more efficient cellular immune effects than those of the inactivated vaccine. Similar results were obtained in a study conducted by Jin Xu *et al*. in CPV VLPs; CPV VP2 VLPs produced better cellular immune responses than inactivated CPV, the response of T lymphocyte proliferation in mice immunized with CPV was higher than that in mice immunized with CPV VLPs, but the percentage of $CD4^+$ or $CD8^+$ T-cells in mice immunized with CPV VLPs was higher than that in mice immunized with CPV virion (Xu *et al.,* 2014). This result suggested that CPV VLPs could induce a more effective cell-mediated immune response against CPV infections compared with CPV virion. Among T cell subsets, although $CD8^+$ T cells, which exert cytotoxic effects on virus-infected cells, are the main effector cells, $CD4^+$ T cells play an pivotal role in the maintenance of the proliferation and differentiation of B cells into plasma cells, the promotion of antibody formation, and the facilitation of the differentiation of $CD8^+$ T cells into cytotoxic T cells (Jhon, 2007). Studies of B cell- and $CD8^+$ T cell-deficient mouse models have found that $CD4^+$ T cells can also be effector cells (Arita *et al.*, 2005). Based on the types of cytokines produced by $CD4^+$ T cells, $CD4^+$ T cells can be divided into Th1 and Th2 cells. Th1 cells mainly secrete IL-2, IFN-γ, and TNF-β, and Th2 cells mainly secrete IL-4, IL-5, and IL-6. The main biological roles of $CD4^+$ T cells are mediated by Th1- and Th2-released cytokines, which are involved in the immune regulation process. Th1 cells are mainly involved in cytotoxicity as well as local inflammatory responses, cellular immune and delayed-type hypersensitivity and can help B cells produce specific antibodies. VLPs provide spatial structures for the display of conformational epitopes, such that VLPs can better simulate the natural structure of the virus and enhance the ability to induce the production of neutralizing antibodies. It has been shown that VLPs can produce effective immune stimulation in the absence of adjuvants. VLPs themselves have an adjuvant effect, and suitably sized VLPs can be presented by dendritic cells (DCs) with major histocompatibility complex (MHC) class II and directly promote dendritic cell maturation and migration, which is the basis for the activation of innate immunity. Exogenous VLPs can also be endocytosed and activate $CD8^+$ T cells via the MHC class I pathway, which is the basis for the removal of intracellular pathogens. One important advantage of VLPs is that VLPs can target dendritic cells, and this targeting feature is the basis for the activation of innate immunity and adaptive immunity. Therefore, VLPs that have targeting abilities are more advantageous as vaccines. In addition, the $CD4^+/CD8^+$ ratios of all of the test groups were in the normal rang of 1.5-2.0 (Grgacic & Anderson, 2006). Similar immune responses have been demonstrated by several types of VLP vaccines, including those derived from the influenza virus, human immunodeficiency virus type 1, human

hepatitis B virus and papilloma virus (Schimbeck *et al*., 1996 (Tsunetsugu *et al*., 2003; Garcia *et al*., 2007). Most of the strong Th1-specific type of immune responses are realized by the reaction between VLPs and DCs, resulting in the stimulation of $CD8^+$ T cells (Tsunetsugu *et al.,* 2003; Lonz *et al.,* 2001; Song *et al*., 2010). The Th1 cellular immune response is necessary to control pathogens inside cells. Ye *et al*. transfected Sf9 insect cells with recombinant baculoviruses (rBV-VP40 or rBV-GP) expressing the Ebola virus VP40 or GP and prepared high levels of VLPs. When these VLPs were used to immunize mice, the GP-specific antibodies produced by the immunized mice were mostly of the IgG2a subtype, suggesting that GP VLPs induced Th1-biased immune responses (Ye *et al.,* 2006).

Changes in the antibody titer and lymphocyte proliferation reflected the humoral and cellular immune statuses, respectively, in animal organs. Merz *et al* (1981) reported that the humoral and cellular immunity in the host plays a pivotal role in the defense against infectious diseases. In this study, we used either inactivated PPV vaccine or VP2 VLPs to immunize gilts and compared the cellular immune levels triggered by inactivated PPV vaccine and by VP2 VLPs by measuring the HI antibody titers of the immunized gilts, detecting cytokine secretion, and examining the proliferation of $CD4^+$ and $CD8^+$ cells (Merz *et al.,* 1981).

CONCLUSION

In summary, the inactivated PPV vaccine induced the upregulation of the Th1 cell subset and IFN-γ and IL-2 cytokine levels in the blood. In addition, the flow cytometry findings of this study were consistent with the cytokine results, further proving that VP2 VLPs also tend to trigger a Th1 immune response.

The results showed that the inactivated PPV vaccine and VP2 VLPs were both capable of inducing humoral and cellular immunity. However, VP2 VLPs lead to better immune responses in the experimental gilts than the inactivated vaccine in terms of humoral and cellular immunity. VLPs do not contain nucleic acids, cannot replicate autonomously, are not contagious, can efficiently induce humoral and cellular immune responses, and may represent a new type of vaccine. This study also showed that PPV VP2 VLPs may be a new protein subunit vaccine candidate.

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